

# The interaction of the bisphosphorylated N-terminal arm of cardiac troponin I-A <sup>31</sup>P-NMR study

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**Abstract** Cardiac troponin I, the inhibitory subunit of the heterotrimeric cardiac troponin (cTn) complex is phosphorylated by protein kinase A at two serine residues located in its heart-specific N-terminal extension. This flexible arm interacts at different sites within cTn dependent on its phosphorylation degree. Bisphosphorylation is known to induce conformational changes within cTnI which finally lead to a reduction of the calcium affinity of cTnC. However, as we show here, the bisphosphorylated cTnI arm does not interact with cTnC, but with cTnT and/or cTnI. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

**Key words:** <sup>31</sup>P-Nuclear magnetic resonance; Cardiac troponin; Cardiac troponin I; Cardiac troponin C; Cardiac troponin T

## 1. Introduction

The inhibitory subunit (cTnI) of the heterotrimeric cardiac troponin complex (cTn) is phosphorylated upon β-adrenergic stimulation by protein kinase A (PKA) [1]. Phosphorylation occurs at serine 22 and 23 (human sequence) [2], which are located in the 32 amino acid long flexible N-terminal extension of cTnI. Sequential phosphorylation and sequential dephosphorylation by protein phosphatase 2A (PP2A) lead to four cTnI forms, one bis- and non-phosphorylated form and two monophosphorylated forms [3,4]. Bisphosphorylation is known to reduce the calcium affinity of cardiac troponin C (cTnC) [5,6] and, as shown recently, reduces the maximal actomyosin ATPase activity [7]. The molecular mechanism is not known. Upon phosphorylation conformational changes occur in cTn, which seem to be accompanied by movements

of the flexible N-terminal cTnI arm. Liao et al. [8] and Dong et al. [9] described a conformational change in cTnI to a more globular shape due to bisphosphorylation at Ser22 and 23. We observed a change in the secondary structure in isolated cTnI upon monophosphorylation at either serine residue using circular dichroism (CD) spectroscopy [10]. Furthermore, monophosphorylation and, to an even larger extent, bisphosphorylation reduce the affinity of cTnI towards the other cTn subunits [11], also indicating conformational changes within cTnI. <sup>31</sup>P-Nuclear magnetic resonance (NMR) spectra of cTn revealed that phosphate groups of mono- and bisphosphorylated cTnI are located in different chemical environments [12]. In the monophospho form, phosphoserine residue (PSer)23, the phosphate group interacts with a preceding arginine side-chain, thus stabilising the reversed turn formed by the phosphorylation region [13]. In monophosphorylated PSer22 the phosphate group is in a nearly identical chemical environment as free phosphoserine indicating that the phosphate group can rotate freely and does not interact with other groups [13]. Thus, in both monophosphorylated forms the phosphorylation region does not interact either with the body of cTnI or with cTnT or cTnC. When cTnI is bisphosphorylated <sup>31</sup>P-NMR measurements reveal a weak interaction of the phosphorylation region within cTn [12–14]. An interaction site in the N-terminal domain (N-DOM) of cTnC would allow the direct influence of calcium binding properties of cTnC. However, as we will show here, the bisphosphorylated N-terminal extension of cTnI does not interact with cTnC, but with cTnT and/or cTnI.

## 2. Materials and methods

### 2.1. Construction of plasmids

The cDNAs encoding human cardiac troponin C (hcTnC) and the major isoform of hcTnT were derived from clone TC1 [15] and from clone HCTNT2 [16], respectively. The cDNA for hcTnC was subcloned into the pET3c vector [13] and the one for hcTnT into the pSBETc vector [17,18] thus serving as templates for the generation of the hcTnC domains (hcTnC-N-DOM and hcTnC-C-DOM) and the C-terminal domain of hcTnT (hcTnT-C-DOM), respectively. The primers for the generation of the hcTnC-N-DOM construct were 5'-GAA TTC ATA TGG ATG ACA TCT ACA AGG CTG-3' (sense) and 5'-AAG CTT GGA TCC CTA AGA TTT CCC TTT GCT GTC GTC C-3' (antisense); those for the hcTnC-C-DOM construct were 5'-GAA TTC ATA TGG TTC GGT GCA TGA AGG ACG-3' (sense) and 5'-AAG CTT GGA TCC CTA CTC CAC ACC CTT CAT GAA CTC-3' (antisense) and those for the hcTnT-C-DOM construct were 5'-GCG CGA ACA TAT GGG GGG TTA CAT CCA GAA GCA G-3' (sense) and 5'-TGA GGA TCC TCA TTT CCA GCG CCC GGT GAC TT-3' (antisense). All sense and antisense primers contained the *Nde*I and the *Bam*HI restriction sites, respectively, for subcloning into the *Nde*I-*Bam*HI site of the pSBETc vector. The sequence of all derived clones was confirmed by auto-

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**Abbreviations:** ESI-MS, electrospray ionisation-mass spectrometry; CD, circular dichroism; C-DOM, C-terminal domain; hcTn, human cardiac troponin; hcTnC, human cardiac troponin C; hcTnI, human cardiac troponin I; hcTnT, human cardiac troponin T; IEF, isoelectric focusing; N-DOM, N-terminal domain; NMR, nuclear magnetic resonance; MOPS, 3-morpholino-propane sulfonic acid; PKA, cAMP-dependent protein kinase (protein kinase A); PP2A, protein phosphatase 2A; RMS, root mean square; PSer, phosphoserine residue; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; WT, wild type

mated sequencing with the Applied Biosystems 373 DNA Sequencer using the ABI *Taq* DyeDeoxy terminator cycle sequencing ready reaction kit.

## 2.2. Protein expression

cTn subunits and their constructs were expressed in *Escherichia coli* BL21(DE3). Cells were grown in NZCYM medium with 50 µg/ml ampicillin (for hcTnI-wild type (WT), hcTnC-WT) or 50 µg/ml kanamycin (for hcTnT-WT, hcTnT-C-DOM, hcTnC-N-DOM and hcTnC-C-DOM) until OD<sub>600</sub> = 0.6. 4 h after induction with 1 ml of 0.4 M isopropyl-β-thiogalactoside solution/l medium cells were harvested by centrifugation and stored at –20°C. Expression was checked with sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Sambrook et al. [19].

## 2.3. Protein purification

hcTnI-WT and hcTnC-WT were purified as described by Reiffert et al. [11] and Babu et al. [20], respectively. For purification of hcTnT-WT, the pellet obtained from a 3 l culture was resuspended in 20 ml 25 mM Tris–HCl, pH 7.5, 20% (w/v) sucrose, 1 mM EDTA, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 0.2 M NaCl, 5 µg/ml *N*-tosyl-L-phenylalanine chloromethyl ketone and 5 µg/ml *N*-*p*-tosyl-L-lysine chloromethyl ketone and stored at –80°C. Cells were disrupted by using a French Press (Aminco). The suspension was incubated at 4°C for 1 h after adding pure urea (final concentration: 5 M) and Triton X-100 (final concentration: 0.1% (v/v)). After centrifugation (1 h, 4°C, 27 000 × g) the supernatant was diluted 10× with 50 mM Tris–HCl, pH 7.5, 5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT) and 5 M urea and subsequently was incubated at 4°C for 2 h with DE52 material (Whatman) equilibrated with the same buffer. After washing off unbound proteins, the suspension was poured into a column and bound protein was eluted with 1 M NaCl added to the buffer. The protein containing fractions were applied onto 400 ml G75 material (Pharmacia) equilibrated with 50 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 2 mM DTT. After elution the purity of collected hcTnT fractions was checked by SDS–PAGE. hcTnC-N-DOM comprising amino acids 1–93 was purified from a bacterial pellet obtained from a 2 l culture following the procedure as described for hcTnC-WT. hcTnC-C-DOM (amino acids 81–161) was purified from pellets obtained from a 6 l culture. Cells were lysed in 90 ml 50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.5 mM PMSF, 25% (w/v) sucrose and 1 mM DTT by using the French Press. After centrifugation (27 000 × g, 4°C, 30 min), urea, MgCl<sub>2</sub> and KCl were added to the supernatant to final concentrations of 8 M, 2 mM and 50 mM, respectively. Urea was removed step-wise and purification was performed as described for hcTnC-WT. For purification of hcTnT-C-DOM comprising amino acids 186–291, cells obtained from 2 l bacterial culture were disrupted in 50 mM Tris–HCl, pH 7.5, 5 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub> and 1 mM DTT by using the French Press. Ammonium sulphate was added to the suspension up to a final concentration of 22.5% (w/v). After stirring for 1 h at 4°C and centrifugation (4°C, 20 000 × g, 30 min) ammonium sulphate was added to the supernatant up to a final concentration of 62% (w/v) and the incubation and centrifugation steps were repeated. The pellet was resuspended in 50 mM Tris–HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 1 mM DTT and pre-purified by cation exchange chromatography as on CM52-material (Whatman) as described for the purification of hcTnT-WT. Collected protein fractions were dialysed in 1 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.9, 100 mM KCl, 2 mM DTT and then applied onto 100 ml hydroxyapatite, equilibrated with the same phosphate buffer. Protein was eluted with 400 mM K<sub>2</sub>HPO<sub>4</sub>, 100 mM KCl, 2 mM DTT.

The purity of all proteins and calcium-dependent mobility of hcTnC-WT, hcTnC-C-DOM and hcTnC-C-DOM were checked by SDS–PAGE. The proteins except hcTnI-WT were stored at –20°C. hcTnI-WT was kept on ice.

## 2.4. Mass determination

The mass of all mutant proteins was determined using electrospray ionisation-mass spectrometry (ESI-MS), TSQ 7000 (Finnigan). During 2 s ions with a mass/charge ratio of 200–2500 were measured. All mass/charge data were analysed by the BioMass software [21].

## 2.5. Phosphorylation of hcTnI-WT

200 nmol of hcTnI-WT was phosphorylated with 200 µU/ml of the recombinant catalytic subunit of PKA [22] for 1 h at 30°C in 20 mM 3-morpholino-propane sulfonic acid (MOPS), pH 7.0, 0.3 M KCl,

5 mM MgCl<sub>2</sub>, 2 mM DTT and 1 mM ATP. The phosphorylation degree was controlled by isoelectric focusing (IEF) and subsequent scanning densitometry according to Ardelt et al. [23]. The total amount of protein bound phosphate was determined according to Stull and Buss [24].

## 2.6. Reconstitution of the various troponin complexes

hcTn complexes were formed by combining recombinant hcTnI-WT, hcTnT (hcTnT-WT or hcTnT-C-DOM) and hcTnC (hcTnC-WT, hcTnC-N-DOM or hcTnC-C-DOM) in a molar ratio of 1:1:1 in 50 mM Tris, pH 7.5, 6 M urea, 0.5 M NaCl, 5 mM CaCl<sub>2</sub> and 5 mM DTT. After incubation for 2 h at room temperature urea was reduced step-wise from 6 to 4 to 2 and finally to 0 M in the same buffer with 1 M KCl. Then the KCl concentration was reduced to 0.3 M. Non-complexed subunits and heterodimers were removed by gel filtration on 400 ml Sephadex G-75 with 10 mM MOPS, pH 7.0, 0.5 M KCl, 1.5 mM CaCl<sub>2</sub> and 1 mM DTT. The ratio of subunits present in the various troponin complexes were controlled by SDS–PAGE and by analytical gel filtration on Sephadex 200 PC3.2/30 by using the Smart-system (Pharmacia). Calibration was performed by using the 'High/Low Molecular Weight Gel Calibration Kit' (Pharmacia).

## 2.7. Molecular modelling

For molecular modelling INSIGHT (User Guides 1995 MSI, San Diego, CA, USA) was used on a Silicon Graphics Indigo2. The solution structure of cTnC [25] was used as template to generate cTnC-N-DOM and cTnC-C-DOM. Coordinates were obtained from the Brookhaven Data Bank (entry code: 1AJ4).

## 2.8. <sup>31</sup>P-NMR spectroscopy

The reconstituted Tn complexes were dialysed in 10 mM NH<sub>4</sub>HCO<sub>3</sub> and dried in vacuo. The dried proteins were dissolved in 700 µl D<sub>2</sub>O containing 20 mM MOPS, pH 7.0, 0.5 M KCl and 2 mM DTT and either 100 µM CaCl<sub>2</sub> or 1 mM EGTA. The pH was not corrected for D<sub>2</sub>O. pH was controlled before and after each measurement. Since small pH differences (even <0.1 pH-units) result in changes of the chemical shift of <sup>31</sup>P-NMR signals [12], inorganic phosphate dissolved in the same buffer was added to each sample as an internal pH standard. For assignment of the <sup>31</sup>P-NMR-signals in different spectra the dependence of the chemical shifts on pH of P<sub>i</sub> and phosphoserine alone or added to the cTn solutions was used [12]. Spectra were recorded on a 600 MHz (proton frequency) Bruker NMR-spectrometer at room temperature. Chemical shifts are quoted relative to external 85% H<sub>3</sub>PO<sub>4</sub> in H<sub>2</sub>O.

## 3. Results

The masses of the WT and mutant proteins determined by ESI-MS coincide with the masses calculated from the amino acid sequences (Table 1). The correct size of the hcTn subunits, their constructs and the purity of the isolated proteins have additionally been verified by SDS–PAGE (Fig. 1). Large

Table 1  
Characteristics of cTn complexes and their subunits

Protein	Mass (kDa)		Retention time (min) <sup>a</sup>
	determined	calculated <sup>b</sup>	
hcTnT-WT	34.5	34.6	40.4
hcTnI-WT	24.0	24.0	42.2
hcTnC-WT	18.4	18.4	42.8
hcTn	n.d.	77.0	32.9
hcTnC-N-DOM	10.5	10.4	47.9
hcTn-hcTnC-N-DOM	n.d.	69.0	34.3
hcTnC-C-DOM	9.3	9.3	48.6
hcTn-hcTnC-C-DOM	n.d.	67.9	35.4
hcTnT-C-DOM	12.5	12.6	46.6
hcTn-hcTnT-C-DOM	n.d.	55	36.7

n.d., not determined.

<sup>a</sup>Retention times were determined twice, mean values are given, a difference in retention time of 0.3 min is significant.

<sup>b</sup>Based on the cDNA sequence.

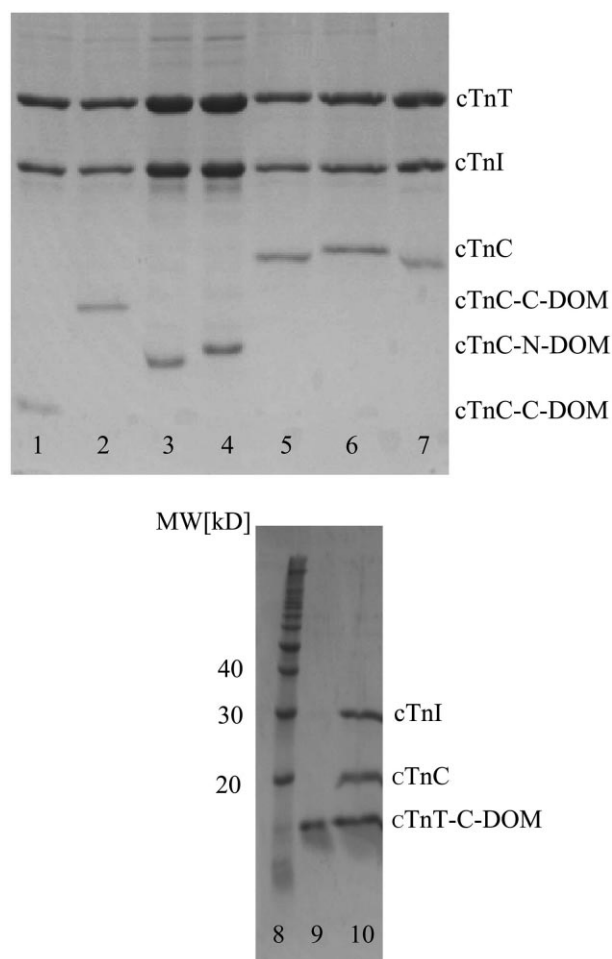


Fig. 1. SDS-PAGE of different cTn complexes, calcium-saturated and calcium-free. In lane 1 and 2, cTn complexes containing hcTnC-C-DOM calcium-saturated and calcium-free, respectively, have been applied. Separation of cTn complexes containing hcTnC-N-DOM in calcium or in EGTA are shown in lane 3 and 4, respectively. As a control hcTn-WT has been applied in calcium or EGTA containing buffer on lane 5 and 6, respectively. As standard, bovine cardiac troponin has been applied on lane 7. A molecular weight marker, cTnT-C-DOM and cTn containing cTnT-C-DOM are applied on lane 8, 9 and 10, respectively. In all lanes about 10  $\mu$ g of protein has been applied.

changes in electrophoretic mobility dependent on calcium saturation was observed for hcTnC-C-DOM (Fig. 1, lanes 1 and 2), only a small effect on mobility was obtained using calcium-free and calcium-saturated hcTnC-N-DOM and cTnC-WT (Fig. 1, lanes 3–6).

The formation of cTn complexes containing the subunits in a molar ratio of 1:1:1 had been verified by analytical gel filtration which yielded a single symmetrical peak at a retention time (Table 1) consistent with the molecular mass of the various complexes. These complexes were phosphorylated by PKA and the phosphorylation degree of cTnI was controlled by IEF (Fig. 2, inserts). The intensity ratio of the mono- and bisphospho-cTnI bands, obtained by scanning densitometry, is comparable to the ratio of the peak areas of the appropriate  $^{31}$ P-NMR signals (not shown). Only those cTn constructs which contained either a mixture of mono- and bisphosphorylated or exclusively bisphosphorylated cTnI were used for  $^{31}$ P-NMR measurements. Over-phosphorylated complexes, which

were obtained seldom, were rejected. In the  $^{31}$ P-NMR spectra of the selected cTn complexes no other signals than those for P-Ser22 and 23 have been detected indicating that only Ser22 and 23 have been phosphorylated by PKA.

$^{31}$ P-NMR spectra of native bovine cTn and of correctly reconstituted hcTn composed of WT subunits both containing a mixture of different cTnI phosphoforms typically show three signals comparable to those in Fig. 2A,B (see also chemical shifts in Table 2). As described formerly for the native protein [12] one signal each had been assigned to the monophospho forms, P-Ser23 (3.91 ppm) and P-Ser22 (3.77 ppm) and one signal (3.51 ppm) to both phosphate groups in bisphosphorylated cTnI (bcTn, Table 2). The difference in chemical shift is due to different apparent  $pK_a$  values of the phosphate groups in the mono- and bisphospho-cTnI forms [12]. This indicates that the phosphate groups are located in different chemical environments. The phosphate group in the monophospho form P-Ser22 behaves like free phosphoserine, the one bound to Ser23 interacts with the preceding arginine side chain present in the consensus sequence for PKA resulting in a chemical shift to higher ppm values [12].

Upon bisphosphorylation both signals shift to a nearly identical lower ppm value (Table 2). This signal is observed exclusively when bisphosphorylated cTnI is present in a correctly folded heterotrimeric cTn complex, not in binary complexes or in the isolated cTnI subunit. Therefore, it represents the interaction of the phosphorylation region in the N-terminal arm of cTnI with another cTn subunit [12] and had been called the 'interaction signal' (IS).

To reveal if cTnC is the interaction partner for the bisphosphorylated cTnI arm, hcTn complexes containing either the N-terminal or C-terminal part of hcTnC have been examined by  $^{31}$ P-NMR spectroscopy (Fig. 2A,B). Since both complexes have not been completely bisphosphorylated as shown by IEF (see insert Fig. 2B), signals for each monophospho form at 3.71 and 3.49 ppm (spectrum A; Table 2) and 3.85 and 3.68 ppm (spectrum B; Table 2) have been obtained. The differences in chemical shift of the P-Ser23 and P-Ser22 signals in the two spectra, A and B, are due to minor differences in pH as can be seen by concomitant shifts of the  $P_i$  signal (Table 2). This is also true for the IS which surprisingly has been observed with both cTn complexes (3.05 ppm (Fig. 2A), 3.36 ppm (Fig. 2B); Table 2). The presence of the IS in both spectra indicates that either the bisphosphorylated N-terminal extension does not interact with cTnC or the N-terminal and C-terminal halves replace each other in the troponin complex. Thus, the backbones of the two cTnC halves were superimposed and root mean square (RMS) fluctuations averaged

Table 2  
Chemical shifts of the  $^{31}$ P-NMR signals

Protein	Chemical shift in ppm			
	P-Ser23	P-Ser22	P-Ser22/23	$P_i$
bcTn	3.91	3.77	3.51	2.00
hcTn-cTnC-N-DOM	3.71	3.49	3.05	1.55
hcTn-cTnC-C-DOM	3.85	3.68	3.36	1.81
hcTn-cTnT-C-DOM	–	–	3.40	1.85

bcTn means cardiac troponin, isolated from bovine heart according to Beier et al. [14],  $P_i$  is inorganic phosphate, P-Ser22 and P-Ser23 designate the signals resulting from the monophospho forms of cTnI and P-Ser22/23 the signal obtained with the bisphosphorylated protein.

over the backbone atoms were calculated. The two halves are clearly different ( $\text{RMS} > 12 \text{ \AA}$ ; Fig. 3). Also the orientation of the central helix, which is involved in the binding of cTnI, is completely different in both cTnC halves upon optimal superimposition of the two EF-hands. Additionally, only cTn complexes have been formed which contain 1 mol cTnC-N- or -C-DOM per 1 mol of cTnT and cTnI as shown by analytical gel

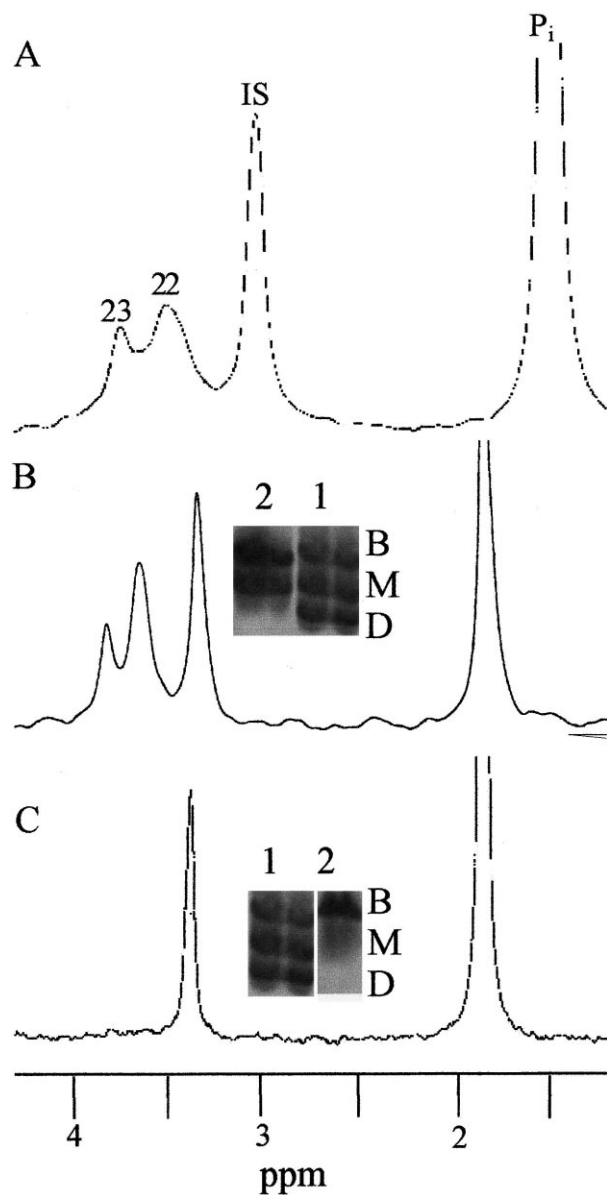


Fig. 2.  $^{31}\text{P}$ -NMR measurements of the various cTn complexes containing cTnI phosphorylated by PKA.  $^{31}\text{P}$ -NMR spectra have been recorded with a spectral width of 2332 Hz. As internal pH standard inorganic phosphate ( $\text{P}_i$ ) was used. A: 15 mg cTn with cTnC-N-DOM (1.8 mol P/mol protein; 48 000 scans) in NMR- buffer; B: 20 mg cTn with cTnC-C-DOM (1.6 mol P/mol protein; 30 000 scans); C: 15 mg cTn with cTnT-C-DOM (2.0 mol P/mol protein, 11 080 scans). IS designates the interaction signal and 23, 22 phosphoserines in position 23 or 22. The inserts show IEF gels, where 4  $\mu\text{l}$  cTnI (10  $\mu\text{g}$ ) isolated from cTn complexes by high performance liquid chromatography as described by Swiderek et al. [30] have been applied (lanes 2). As standard, bovine cTnI isolated from bovine heart [14] has been applied (lanes 1). B, M and D designate bisphosphorylated, monophosphorylated and non-phosphorylated cTnI, respectively.

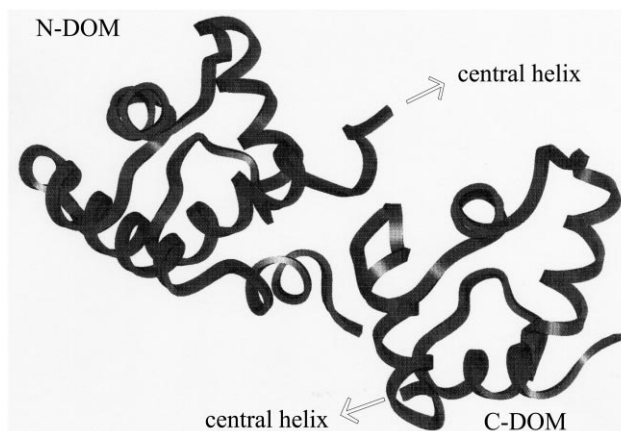


Fig. 3. Superimposition of cTnC-N-DOM and cTnC-C-DOM. Superimposed backbones of hcTnC-N-DOM and hcTnC-C-DOM are dragged apart and presented as ribbon.

filtration (compare retention times in Table 1). Thus it can be concluded that the bisphosphorylated cTnI extension interacts with a cTn subunit other than cTnC.

To decide in which region of cTn the interaction might occur, a N-terminally truncated hcTnT, hcTnT-C-DOM (amino acids 185–291), was reconstituted with hcTnI-WT and hcTnC-WT to a ternary hcTn complex. The  $^{31}\text{P}$ -NMR spectrum again shows the IS (Fig. 2C) indicating that an interaction might occur within the sequence stretch of 185–291 of cTnT. Furthermore, all spectra recorded with cTn complexes in calcium-saturated and calcium-free state were identical (therefore only one set of data is shown in Fig. 2).

#### 4. Discussion

$^{31}\text{P}$ -NMR spectroscopy is an excellent tool to observe directly each single phosphate group in a protein when present in different chemical environments. Using this method we characterised the different phospho forms of cTnI within the complex, which together with data from other experiments led to the conclusion that the heart-specific N-terminal extension of cTnI is a flexible arm which alters the interaction partner within the cTn molecule dependent on its phosphorylation degree [12–14]. In contrast to the monophosphorylated cTnI arm, both, the non- and the bisphosphorylated cTnI arm seem to make contacts within the cTn complex. As shown by CD spectroscopy changes in the backbone of isolated cTnI were observed upon monophosphorylation, which led to the conclusion that the non-phosphorylated extension interacts with cTnI itself and is released after monophosphorylation [10]. Other groups described an interaction of the non-phosphorylated cTnI-N-terminal arm with the C-DOM of cTnC using cTnC and fragments of cTnI or vice versa [26–28]. These contradictions are probably caused by the use of isolated subunits on the one hand and binary complexes with peptides on the other.

In accordance with these groups [26–28], we could not detect an interaction of the bisphosphorylated cTnI arm with cTnC in the heterotrimeric cTn complex as shown here. As with native cTn the IS has been obtained with both cTn constructs containing either cTnC-N-DOM or cTnC-C-DOM. If the interaction partner would be, for example, cTnC-N-DOM,

one would expect the IS in the  $^{31}\text{P}$ -NMR spectrum obtained with the cTn complex containing cTnC-N-DOM, but not in the spectrum with cTn containing cTnC-C-DOM. Vice versa the IS should be observed in the spectra of cTn with cTnC-C-DOM, but not in the spectrum of cTn with cTnC-N-DOM, if the interaction partner is the C-DOM of cTnC.

The attractive conclusion of Gaponenko et al. and Abbott et al. [27,28] that the release of the cTnI arm by phosphorylation of cTnI at Ser22 and 23 directly leads to the reduction of the calcium affinity of cTnC does not take into account that already monophosphorylation releases the cTnI arm [10,12]. Although effects of monophosphorylation on the affinity of cTnI to cTnT and cTnC [11] and on the actomyosin ATPase activity [7] have been described, an influence on cTnC-calcium affinity could be detected exclusively upon bisphosphorylation of cTnI [5–7,10]. Therefore changes induced by bisphosphorylation within the troponin complex including the interaction of the bisphosphorylated cTnI arm are decisive to alter the calcium affinity of cTnC. These observations and the results presented here favour an indirect propagation of the PKA signal as has also been proposed by Chandra et al. [29]. They postulated an interaction of the bisphosphorylated cTnI arm with cTnI itself. Such an interaction cannot be excluded by the  $^{31}\text{P}$ -NMR experiments presented here, but might also occur within the C-DOM of cTnT (186–261). Since chemical shift or half line width of the IS is not altered upon calcium binding or release, the interaction takes place in a calcium insensitive region. Furthermore, binary TI or IC complexes are not sufficient to provide the ‘correct’ environment allowing the interaction of the bisphosphorylated cTnI arm. [12], but a ternary complex with either the C-terminal part of cTnT or one of the two cTnC constructs is.

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